A Research Note

Some Observations on a Perigo-Type Inhibition of *Clostridium botulinum* in a Simplified Medium

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ABSTRACT

A rapid and sensitive assay for Perigo factor was developed using a medium of 0.5% yeast extract and tryptone, 0.2% glucose, 0.12% K_2HPO_4 and 0.1% cysteine • HCl or sodium thioglycollate and vegetative cells of *Clostridium botulinum* type A. Yeast extract or tryptone, together with a reducing agent (cysteine, sodium thioglycollate, or glucose autoclaved with the medium), produced a Perigo inhibitor when autoclaved at 15 psi for 15 min with $NaNO_2$. Tryptone was more active than yeast extract as a source of the Perigo inhibitor; of the reducing agents tested cysteine was more effective in producing Perigo-type inhibition than thioglycollate and either was better than glucose autoclaved with the medium.

A potent inhibitor of vegetative cells of *Clostridium sporogenes* was produced by heating nitrite in a complex medium composed of tryptone, peptone, beef extract (Lab-Lemco), yeast extract, NaCl, K_2HPO_4, glucose, soluble starch, sodium thioglycollate, and brom cresol purple [Perigo et al. (*3*)]. The study reported here was undertaken to develop a more rapid assay for such inhibitors—the original procedure of Perigo et al. requiring 10 days of incubation. The assay medium was then used to identify the component(s) of the medium involved in the formation of the Perigo inhibitor.

MATERIALS AND METHODS

The basic solution used in developing the simpler assay medium contained the chemically defined components of Perigo's medium: 0.5% NaCl; 0.25% K_2HPO_4; 0.2% glucose; 0.1% starch; 0.1% sodium thioglycollate, and 0.008% brom cresol purple. To this basal solution were added 0.5% concentrations of the four crude materials; beef extract, yeast extract, peptone, and tryptone, either individually or in various combinations. A Seitz-filtered, 1.6% solution of $NaNO_2$ was diluted 1:1 serially with sterile water and 0.2 ml of each dilution was added to 5 ml of the medium either before or after autoclaving at 15 psi for 15 min to give final concentrations (ppm) of $NaNO_2$ of 640, 320, 160, 80, etc. to 1.25.

Culture and inoculum

Stock cultures of *Clostridium botulinum* type A (#B1218 from the Northern Regional Research Center, USDA, Peoria, Ill.) were maintained in a cooked meat medium and transferred by needle into a medium containing 0.5% each of tryptone, yeast extract, beef extract, and peptone, 0.2% glucose, 0.1% sodium thioglycollate, and 0.12% K_2HPO_4, at pH 6.6, with an overlay of vaspar. These were incubated 20-24 h at 35 C. The inoculum for the assay was one drop of this culture, previously diluted 1-50 with the assay medium, to 5 ml of medium in 15 × 125 mm culture tubes for a final cell concentration of about 300/ml. Duplicate tubes were incubated at 35 C for 24 h. Further incubation up to one week did not appreciably change the results. Initially, an atmospheric incubator was used while later experiments were carried out in a controlled atmosphere incubator evacuated to 20-27 in. mercury with the vacuum being replaced with nitrogen gas. Growth in the latter system was slightly faster.

Perigo index

Inhibition of growth of the organism was expressed as the lowest concentration of nitrite, in ppm, in the tube which did not allow growth of the organism with turbidity being used as the criterion. The ratio of this minimum inhibitory concentration (mic) of the nitrite added after autoclaving to the mic of nitrite autoclaved with the medium was designated for convenience as the Perigo Index (PI). The PI indicates the magnitude of the heated nitrite effect; for example, a PI of 2 indicates that twice as much unheated nitrite was needed for inhibition than nitrite heated with the medium.

RESULTS AND DISCUSSION

Beef extract, added to the basic, chemically defined solution above, did not support growth and only slight growth was obtained in the medium containing peptone alone. Yeast extract or tryptone alone gave better growth while a combination of yeast extract and tryptone gave nearly as good growth as the complete medium. Further studies indicated that NaCl, starch, and brom cresol purple were not necessary for growth, had no effect on growth inhibition and were therefore eliminated from the medium. The final medium for assay of the "Perigo Inhibitor" consisted of 0.5% yeast extract, 0.5% tryptone, 0.2% glucose, 0.12% K_2HPO_4, with either 0.1% sodium thioglycollate or cysteine • HCl. No pH adjustment was needed with the former while neutralization was needed with cysteine.

Effect of reductants

A comparison of 0.1% cysteine and sodium thioglycollate as reducing agents in the medium for production of the Perigo inhibitor is shown in Table 1. Cysteine was more effective, giving a PI of 64 (first line in Table 1—the complete medium) compared to 16 with

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TABLE 1. *Effect of separately autoclaved reducing agents or glucose on the formation of Perigo inhibitor*

Components added after autoclaving	Ratio[a] (mic, ppm)
None (complete medium with cysteine autoclaved)	160/2.5 = 64[b]
Cysteine	40/2.5 = 16
Glucose	160/2.5 = 64
Cysteine and glucose	160/80 = 2
None (complete medium with thioglycollate autoclaved)	80/5 = 16
Thioglycollate	40/5 = 8
Glucose	80/5 = 16
Thioglycollate and glucose	80/80 = 1

[a]$NaNO_2$ after autoclaving/$NaNO_2$ before autoclaving.
[b]Perigo Index.
Incubation was in a N_2 atmosphere.

sodium thioglycollate; however, for reasons of economy and availability, the sodium thioglycollate was generally used. Adding separately autoclaved cysteine to the medium autoclaved without cysteine reduced the PI to 16, whereas similar treatment with sodium thioglycollate decreased the PI to 8. This seemed to indicate that a reducing atmosphere was conducive to formation of the Perigo inhibitor. Addition of separately autoclaved glucose to the the otherwise complete medium had no effect on the PI, but if both glucose and the reducing agent were absent during autoclaving, and added later the Perigo inhibitor was not formed. Autoclaving glucose with the medium evidently provided sufficient reductant for the Perigo effect to develop. There was a decrease in mic for unheated nitrite from 160 for the complete medium to 40 when cysteine was added separately. This indicated that autoclaving without cysteine but with glucose in the medium made the unheated nitrite more effective.

Separately added yeast extract and tryptone

The effect of separately autoclaving other components of the medium on the formation of the Perigo inhibitor is shown in Table 2. The PI for the complete medium was 64, the same as in Table 1, although the mic's for the ratio were higher. This may have been due to the difference in incubation conditions or to variation from different batches of sodium thioglycollate which were shown by Huhtanen and Wasserman (*2*) to influence the mic's obtained although the PI's were not appreciably influenced. The absence of yeast extract or tryptone from the medium during heating led to a decrease in the Perigo index from 64 to 16. If in addition to yeast extract or tryptone, cysteine was also added after autoclaving the Perigo index was reduced even further to 2. When $NaNO_2$ was autoclaved in a neutral solution of glucose and K_2HPO_4, with the other ingredients (cysteine, yeast extract, and tryptone) added after autoclaving, the PI of 0.5 indicated that some nitrite was probably destroyed during the heating. The results in Table 2 indicate that Perigo inhibitor is formed when either yeast extract or tryptone, or both, together with a reducing agent and nitrite are autoclaved together. The formation of such a Perigo inhibitor from tryptone and nitrite was also recently reported by Grever (*1*).

TABLE 2. *Effect of separately autoclaved cysteine, yeast extract, or tryptone on the formation of Perigo inhibitor*

Component added after autoclaving	Ratio[a] (mic, ppm)
None (complete medium)	320/5 = 64[b]
Cysteine	320/80 = 4
Yeast extract	320/20 = 16
Yeast extract and cysteine	320/160 = 2
Tryptone	160/10 = 16
Tryptone and cysteine	320/160 = 2
Tryptone and yeast extract	80/40 = 2
Tryptone and yeast extract and cysteine	160/320 = 0.5

[a]$NaNO_2$ after autoclaving/$NaNO_2$ before autoclaving.
[b]Perigo Index.
Incubation was in an atmospheric incubator.

REFERENCES

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